The GntR-Type Regulators GtrA and GtrB Affect Cell Growth and Nodulation of *Sinorhizobium meliloti*

Yi Wang†, Ai-Min Chen†, Ai-Yuan Yu†, Li Luo†, Guan-Qiao Yu†, Jia-Bi Zhu†, and Yan-Zhang Wang*†

†National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, Shanghai 200032, P. R. China
‡Biological Sciences Department, Lehman College, The City University of New York, New York 104681, USA

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GntR-type transcriptional regulators are involved in the regulation of various biological processes in bacteria, but little is known about their functions in *Sinorhizobium meliloti*. Here, we identified two GntR-type transcriptional regulator genes, gtrA and gtrB, from *S. meliloti* strain 1021. Both the gtrA1 mutant and the gtrB1 mutant had lower growth rates and maximal cell yields on rich and minimal media, as well as lower cell motility on swimming plates, than did the wild-type strain. Both mutants were also symbiotically deficient. Alfalfa plants inoculated with wild-type strain 1021 formed pink elongated nodules on primary roots. In contrast, the plants inoculated with the gtrA1 and gtrB1 mutants formed relatively smaller, round, light pink nodules mainly on lateral roots. During the first 3–4 weeks post-inoculation, the plants inoculated with the gtrA1 and gtrB1 mutants were apparently stunted, with lower levels of nitrogenase activity, but there was a remarkable increase in the number of nodules compared to those inoculated with the wild-type strain. Moreover, the gtrA1 and gtrB1 mutants not only showed delayed nodulation, but also showed markedly reduced nodulation competition. These results demonstrated that both GtrA and GtrB affect cell growth and effective symbiosis of *S. meliloti*. Our work provides new insight into the functions of GntR-like transcriptional regulators.

**Keywords:** GntR-type transcriptional regulators, GtrA, GtrB, cell growth, nodulation

*Sinorhizobium meliloti* is a Gram-negative soil bacterium that is capable of establishing a symbiotic relationship with the alfalfa plant, *Medicago sativa*. A complex interplay between a bacterium and its symbiotic partner leads to the formation of a morphologically specialized plant organ, the nodule, in which *Rhizobia* carry out nitrogen fixation. During the establishment of successful symbiosis, a precise molecular dialogue between symbiotic partners is triggered when root-secreted plant flavonoids act as transcriptional activators of *rhizobium* nodulation (Long, 1996). In the ensuing process, proteins encoded by the activated nod genes aid in the synthesis of rhizobial signal molecules known as nod factors, which play a pivotal role in determining the fate of symbiotic interaction (Denarie *et al*., 1996; Long, 1996). Accumulating evidence has proven that LysR-type transcriptional regulators play a crucial role in an effective symbiotic relationship (Honma and Ausebel, 1987; Mulligan and Long, 1989; Kondorosi *et al*., 1991; Swanson *et al*., 1993; Luo *et al*., 2005). Based on information from the *S. meliloti* sequencing project, it is known that GntR-type transcriptional regulators comprise the second largest family of transcriptional regulators in *S. meliloti* 1021 (Galibert *et al*., 2001). This information suggests that the GntR family of transcriptional regulators can play an important role in symbiosis between *S. meliloti* and alfalfa. However, little is known about the functions of the GntR family of transcriptional regulators in *S. meliloti*.

The GntR family of transcriptional regulators is one of the most prevalent superfamilies of bacterial transcription factors. They are generally composed of an N-terminal winged HTH (helix-turn-helix) domain, followed by a C-terminal domain that can bind a variety of ligands (Fujita and Fujita, 1987; Haydon and Guest, 1991; Rigali *et al*., 2001). Members of the GntR family of proteins are currently clustered into six subfamilies on the basis of C-terminal effector-binding and oligomerization domains, e.g., FadR, HutC, MocR, YtrA, AraR, and PImA (Rigali *et al*., 2001; Lee *et al*., 2003). They can function as activators or repressors, although most of them are repressors. The consensus binding sequence for GntR-like transcriptional regulators is a [5’-(N)yGT(N)xAC (N)y-3’] motif, but the promoters of targeted genes are not highly conserved (Rigali *et al*., 2001).

Most of the GntR-like transcriptional regulators have been reported to be involved diverse metabolism pathways in bacteria; among these GntR-like transcriptional regulators are the FadR, HutC, MocR, and AraR subfamilies (Mota *et al*., 1991; Rigali *et al*., 2001). The YtrA subfamily has been implicated in the ATP binding cassette (ABC) transport system, which is common in prokaryotes (Yoshida *et al*., 2000). The PImA subfamily might regulate plasmid maintenance in *Anabaena* (Lee *et al*., 2003). The AraR subfamily is involved in absorbing and utilizing the carbohy-
drates (Sa-Nogueira et al., 1997; Mota et al., 1999). Recent evidence suggests that some GntR-type transcriptional regulators also control the development of bacteria. DasR in Streptomyces coelicolor regulates the sugar phosphotransferase system and links N-acetylglucosamine metabolism to the control of development (Rigali et al., 2006). AgI3R in S. coelicolor is required for morphogenesis and antibiotic production, and controls transcription of an ABC transporter in response to a carbon source (Hillerich and Westpheling, 2006). DevA is required for development in S. coelicolor (Hoskisson et al., 2006). Mce1R is required for organized granuloma formation, which is both protective to the host and necessary for the persistence of Mycobacterium tuberculosis (Casali et al., 2006). Systematic targeted mutagenesis of Brucella melitensis 16M reveals a major role for GntR regulators in the control of virulence (Haine et al., 2005).

The complete sequencing of the S. meliloti genome revealed 54 putative genes encoding GntR-type transcriptional regulators, which allowed us to conduct a systematic study of the GntR family of transcriptional regulators in S. meliloti (Galibert et al., 2001). We successfully mutated the 54 gntR (GntR family of transcriptional regulators) genes by plasmid insertion mutagenesis and screened for their phenotypes. During this process, we found two gntR mutants to be symbiotically deficient. Here, we reported the characterization of the two gntR genes, gtrA, and gtrB, in S. meliloti 1021. Mutation of gtrA or gtrB not only affects cell growth and maximal cell yield, but also impairs symbiosis with alfalfa plants. Although both mutants were able to form nitrogen-fixing nodules, they varied in terms of the timing of nodules emergence, the progression of nitrogen fixation, the number of nodules, and the nodulation competitiveness.

**Materials and Methods**

**Secondary structure prediction**

Secondary structure prediction was performed by using the compilation of the PSI-pred, Predict Protein, Spro, and Jpred automated prediction programs on the PredictProtein server (http://cubic.bioc.columbia.edu).

**Strains and culture conditions**

The Escherichia coli DH5α strain was used for cloning, MT616 (pRK600) (Cm') was used as the helper strain for conjugation, and S. meliloti 1021 (Str') was used as the wild-type strain (Leigh et al., 1985). E. coli strains were cultured on Luria-Bertani (LB) medium at 37°C, and all S. meliloti strains were cultured in LB/MC (LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM L-CaCl₂) (Leigh et al., 1985). Minimal medium Z-MGS was used to examine the growth requirements of the mutants (Zevenhuizen and Van Neerven, 1993). Agar (1.5%) was used as the solid medium. Antibiotics were supplemented as required at the following concentrations: chloramphenicol, 10 µg/ml; kanamycin, 50 µg/ml; neomycin, 200 µg/ml; and streptomycin, 500 µg/ml.

**Plasmid insertion mutagenesis**

Plasmid insertion mutagenesis was conducted as described by Luo et al. (2005). Suicide plasmid pK19mob2HMB was prepared using the Wizard Plus Midiprep DNA purification system (Promega, USA), digested with restriction enzymes HindIII and BsrGI (New England BioLabs, USA), and purified with the Watson PCR purification kit (Watson Inc., China). The DNA fragments (~0.3 kb) containing the middle part of each putative gntR gene were amplified by PCR from S. meliloti 1021 genomic DNA with primers specific for each gene, and the enzyme cleavage sites for HindIII and BsrGI were introduced into the primer sequences. The primers used to amplify the partial sequence of the gtrA gene (the SmaI open reading frame) were: Primer 1; 5'-GGGTGTGACAGGTTGATGGCTGAATCGGAAG-3' (BsrGI site underlined) and Primer 2; 5'-GGGAAGCTTCCATGTTCCTGAAAGAGA-3' (HindIII site underlined), and the primers used to amplify the partial gtrB gene (the SmaI open reading frame) were: Primer 3; 5'-GCGTGATACAGGGTGATGGCTGAATCGGAAG-3' (BsrGI site underlined) and Primer 4; 5'-GGGAAGCTTCGGCCGCAAGAATGCGTGAAG-3' (HindIII site underlined). A 0.3 kb fragment that had been isolated from the PCR products was digested with HindIII and BsrGI, and was then ligated into pK19mob2HMB precleaved with the same enzymes. The recombinant plasmids were first transformed into DH5α, and were then conjugated into wild-type strain 1021 using MT616 as a helper in a triparental mating. Successful plasmid insertion into the gtrA or gtrB gene was selected on solid LB/MC containing streptomycin and neomycin. Single colonies were streaked twice on the same selective medium. A bacterial culture inoculated from a single colony on the selective medium was lysed with S. meliloti phage qM12. The lysate was then introduced back into wild-type 1021 in order to select for a single mutation of the gtrA or gtrB gene.

Successful insertion was confirmed by PCR using genomic DNA isolated from the mutant S. meliloti cells. PCR was performed with one primer that annealed to the suicide plasmid (primer 5; 5'-CCTGGGCGCTTTCGCTGGCCT-3') and another primer complementary to the putative gtrA and gtrB genes (Primer 1 for the gtrA gene and Primer 3 for the gtrB gene). When the plasmid is inserted into the target gene, a DNA fragment with a predicted size will be amplified by PCR. Otherwise, the integration process will not be successful.

**Swimming behavior assay**

To ensure the synchronicity of the bacterial liquid cultures, all of the strains were routinely initiated from glycerol stocks. Wild-type strain 1021 and mutants were grown to exponential phase at 28°C in LB/MC, subcultured in 5 ml fresh LB/MC with appropriate antibiotics, and grown to early exponential phase (OD₆₀₀=0.15–0.30). Aliquots (2 µl) of the bacterial cultures (~2×10⁷ cells) were dispensed uniformly onto the surfaces of freshly prepared LB/MC agar (0.3% agar) swimming plates, and were then incubated at 28°C for 4 days.

**Growth curve assay**

A single colony of each bacterial strain was inoculated into a 5 ml liquid LB/MC medium with the appropriate antibiotics, and was grown to an OD₆₀₀ of ~2.0 at 28°C. The cultures were then subcultured (1:100) with the same medium, and the cell density was measured by monitoring the optical density